Vloeistof-vloeistof verdeling als onderzoekmethode bij de vergelijking van enige rassen van digitalis purpurea L.

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SAMENVATTING

De samenstelling van het glycosidenmengsel van drie rassen van *Digitalis purpurea* L. werd in verschillende groeistadia onderzocht. Door extractie met chloroform van een waterige oplossing der in het gefermenteerde blad voorkomende glycosiden werden deze in twee groepen verdeeld, een in chloroform oplosbare fractie en een in water oplosbare fractie. De samenstelling van het chloroformextract werd met behulp van de gefractioneerde extractiemethode volgens Craig nader onderzocht. Het gehalte van het in de waterlaag achterblijvende gedeelte van het glycosidenmengsel aan de gezamenlijke aglyconen der A- en der B-serie werd spectrofotometrisch bepaald.

HOOFDSTUK I.

Een overzicht wordt gegeven van de literatuur betreffende de steroïdglycosiden van D. purpurea. Hierin zijn behalve de hartglycosiden ook de digitanolglycosiden en de saponinen betrokken.

HOOFDSTUK II.

Planten van *D. purpurea* L., rassen 15, 20 en F[154, 212], werden gekweekt op de proeftuin te Buitenpost (Fr.). Bladmateriaal van elk der rassen werd in vier groeistadia verzameld, te weten van jonge planten (zie foto, blz. 46), van omstreeks vier maanden oude planten (halfvolwassen rozet; alleen voor de spectrofotometrische bepalingen), van planten aan het einde van het eerste jaar hunner ontwikkeling (volwassen rozet) en van bloeiende planten in het tweede jaar (rozet- + stengelbladeren).

De spectrofotometrische bepaling van de glycosidengehaltes vond plaats met behulp van de kleurreacties met xanthydrol, 3,5-dinitrobenzoaat en pikraat, met de laatste zowel voor als na hydrolyse tot de (anhydro)-geninen. De gitoxigenine-bepaling werd uitgevoerd met het reagens volgens TATTJE[187]. De papierchromatografie vond in hoofdzaak plaats met de door KAISER en medewerkers[23, 53, 190] beschreven vloeistofsystemen.

De bereiding van het ruwe extract, dat als uitgangsstof voor de Craig-verdelingen diende, wordt beschreven.

HOOFDSTUK III.

De ruwe glycosidenmengsels werden aan een Craig-verdeling onderworpen (basisproces, 94 transporten) in het vloeistofsysteem methyleenchloride/benzeen/methanol/water = 13:7:13:7 v/v. De verkregen fracties werden op hun glycosidengehalte geanalyseerd, zowel met 3,5-dinitrobenzoaat als met xanthydrol. Bovendien werd een gewichtscurve opgenomen. Voor de algemene vorm van de verdelingscurven en de plaats der verschillende verbindingen, zie figuur 19; voor papierchromatogrammen van bijbehorende fracties, zie figuur 20. Enige bijzonderheden in de verdelingscurven worden besproken. Als zodanig worden genoemd: *a.* de verbinding OD3; *b.* de afwezigheid van gitaloxine en verodoxine in het ruwe extract tengevolge van de deformylering met loodacetaat; *c.* de mono- en bisdigitoxosiden van verschillende aglyconen; *d.* een bij de rassen 15 en 20 waargenomen verschil in de suiker- en aglyconwaarde voor de gitoxinepiek; *e.* de aanwezigheid van twee nauw met digipurpurine verwante digitanolglycosiden, aangeduid als de verbindingen OD1 en OD2, in de gitoxinepiek bij ras F; *f.* de aanwezigheid van digiproside, voornamelijk in bladmonsters van jonge planten; *g.* het voorkomen van enige digitanolglycosiden aan het einde van de buizenreeks, omstreeks fractie 80.

HOOFDSTUK IV.

Ter nadere identificatie van de bij de verdelingen waargenomen verbindingen werden de meeste hiervan in zuivere toestand afgezonderd. Hiertoe werd gebruik gemaakt van kolomchromatografie (aluminiumoxide; silicagel of cellulosepoeder, geïmpregneerd met formamide), toegepast op verzamelde fracties der Craig-verdelingen. De verkregen verbindingen werden wat betreft hun fysisch-chemische eigenschappen voor zover mogelijk met authentieke monsters vergeleken.

De volgende stoffen werden geïsoleerd (tussen haakjes het piekmaximum): verbinding OD3(7), digitoxine (22), digitoxigenine-bisdigitoxoside en -monodigitoxoside (resp. 28 en 34), gitaloxine (uit een niet met loodacetaat gedeformyleerd extract) (28), digipurpurine (39), gitoxine (54), gitoxigenine-bisdigitoxoside en -monodigitoxoside (resp. 60 en 66), de verbindingen OD1 en OD2 (resp. 52 en 60) en strospeside (68).

Tenslotte is een overzicht van de R_F-waarden van enige suikers (tabel 13) en van een aantal glycosiden en aglyconen (tabel 14) opgenomen.

De resultaten van het onderzoek naar de samenstelling van het glycosidenmengsel van de rassen 15, 20 en F worden medegedeeld. Die van de Craig-verdelingen zijn ten dele grafisch, ten dele in tabelvorm weergegeven. De uitkomsten van de spectrofotometrische gehaltebepalingen in de bladpoeders zijn in een drietal tabellen samengevat.

De samenstelling van het glycosidenmengsel der drie rassen, hoewel onderling sterk verschillend, bleek slechts weinig verandering te vertonen tijdens de onderzochte groeiperioden. De tussen de rassen bestaande verschillen in samenstelling manifesteren zich dus in alle groeistadia op ongeveer gelijke wijze. Deze verschillen komen het duidelijkst tot uitdrukking in de samenstelling van het in chloroform oplosbare gedeelte en wel speciaal wat betreft de hartglycosiden. Voor de digitanolglycosiden worden slechts geringe verschillen tussen de rassen waargenomen. Ook de in water oplosbare fractie vertoont, zowel wat het gehalte als wat de samenstelling betreft, slechts weinig verschil bij de drie rassen.

De rassen 15 en 20 zijn rassen met overwegend B-glycosiden; ras 15 bevat in alle groeistadia meer strospeside en minder gitoxine dan het overigens sterk hieraan verwante ras 20. Ras F verschilt sterk van de beide overige rassen; het in chloroform oplosbare gedeelte van het na fermentatie aanwezige glycosidenmengsel bestaat in alle groeistadia voor omstreeks 70 % uit digitoxine. Een soortgelijk ras is het hier niet nader onderzochte ras Cambridge[154].

De mogelijkheid om door selectie Digitalisrassen te verkrijgen met een sterk verschillende samenstelling van het glycosidenmengsel (vooral tot uiting komend in de in chloroform oplosbare fractie) wordt door dit onderzoek nader bevestigd en de verschillen in samenstelling worden scherp gedefiniëerd.

Ten aanzien van ontwikkelingen in de samenstelling van het glycosidenmengsel tijdens de groei kan het volgende opgemerkt worden.

a. Het glycosidengehalte in het bladpoeder en in het daaruit bereide chloroformextract is het hoogst in het volwassen stadium, het laagst in het jonge stadium en heeft in het bloeiende stadium een tussenliggende waarde. Vroegere waarnemingen door andere auteurs worden hiermede bevestigd. Tevens wordt hierdoor aangetoond dat deze gehalteverschillen geen gevolg zijn van variaties

in de onderlinge verhouding der glycosiden tijdens de ontwikkeling der plant;

b. Beschouwen wij de in de waterlaag achterblijvende glycosiden als percentage van het gehele glycosidenmengsel, dan blijkt dit percentage tijdens de groei af te nemen. Daar de voornaamste suiker in het chloroformextract digitoxose (+glucose) is, in de waterlaag daarentegen digitalose (+glucose), zou deze daling op een verschuiving in de aard der door de plant gesynthetiseerde suikers tijdens de ontwikkeling kunnen wijzen (vgl. LEMLI[142]). Het hogere gehalte aan digiproside in jonge bladeren zou ook langs deze weg verklaard kunnen worden;

c. De ontwikkeling van het glycosidenmengsel verloopt voor de verschillende rassen niet geheel gelijk, ook al bestaat een sterke mate van overeenstemming;

d. De digitanolglycosiden zijn in het jonge en bloeiende stadium relatief belangrijker dan in het volwassen stadium. Dit is in overeenstemming met de zienswijze van TSCHESCHE[139] dat de digitanolglycosiden als bouwsteen bij de biosynthese van de hartglycosiden zouden fungeren, een bewijs voor zijn hypothese kan hieruit evenwel niet worden afgeleid.

SUMMARY

The composition of the glycoside mixture in three strains of *Digitalis purpurea* L. was studied in various growth-stages. By extraction with chloroform of an aqueous solution of the glycosides from fermented leaves, the glycoside mixture was divided into two parts, a chloroform-soluble fraction and a water-soluble fraction. The composition of the chloroform extract was examined by means of the Craig countercurrent distribution. The glycoside mixture remaining in the water layer was investigated by spectrophotometric methods as to its content of genins of the A- and B-series. The question whether differences in composition of the glycoside mixture in strains of D. purpurea, obtained by selective breeding, are maintained during development could thus be answered. Moreover, information could be obtained regarding the development of the glycoside mixture during growth.

CHAPTER I.

A review is given of the literature pertaining to the steroidal glycosides of Digitalis, covering not only the cardiac glycosides but also the digitanol glycosides and the neutral saponins.

CHAPTER II.

Plants of *D. purpurea* L., strains 15, 20 and $F^{154, 212}$ were grown at the experimental garden in Buitenpost, Holland. Leaf material from each strain was collected in four different stages of growth, viz. from young plants (see photograph, p. 46), from plants about four months old (half-mature leaf rosettes; for spectrophotometric determinations only), from plants at the end of their first year development (mature rosettes) and from flowering plants in the second year (rosette + stem leaves). Data of harvest as well as quantities of fresh and dry material appear in table 5.

The spectrophotometric determination of glycoside contents was carried out by means of the colour reactions with xanthydrol, 3,5-dinitrobenzoic acid and picric acid, with the latter both before and after hydrolysis to the (anhydro)-genins. Gitoxigenin was estimated with the reagent described by TATTJE[187]. Paper chro-

matography was carried out mainly with various formamide saturated solvent systems as described by KAISER et al.[23, 53, 190].

The preparation of the crude extract used as starting material for the Craig distributions is described. Prior to extraction the leaf material was fermented during three days at 30° C; as a rule 500 to 750 g of dry leaf powder was worked up. In order to accomplish a deformylation of the formyl glycosides (gitaloxin, verodoxin), lead acetate was always used in quantities excessing the amount needed for a complete clarification of the extracts.

CHAPTER III.

The crude mixture of glycosides (3 to 4 g from 500 g of dry leaf material) was subjected to Craig distribution in the solvent system methylene chloride/benzene/methanol/water = 13:7:13:7 v/v. The number of transfers was 94 (fundamental procedure), the volume of both upper and lower phase 25 ml. In the obtained fractions the glycoside content was determined with 3,5-dinitrobenzoate and xanthydrol reagents. The dry weight of the fractions was determined as well.

The general shape of the distribution curve and the location of the various glycosides is shown in figure 20, paper chromatograms of corresponding fractions are given in figure 21. Some peculiarities in the distribution patterns are discussed. In particular we draw attention to the following features.

a. The compound OD3, a digitanol glycoside we have isolated from the first peaks of the Craig distributions. It probably is formed from diginin during its isolation;

b. Gitaloxin and verodoxin are absent, owing to their deformylation with lead acetate. For the distribution curve of a crude extract which was not deformylated with lead acetate, see figure 21, for paper chromatograms of fractions from this distribution, both before and after treatment with aqueous ammonia, see figure 22;

c. The mono- and bisdigitoxosides of digitoxigenin and gitoxigenin are located to the right of the maxima for the corresponding tridigitoxosides (digitoxin and gitoxin). Their presence could be demonstrated by paper chromatographic comparison with products of the controlled hydrolysis of digitoxin and gitoxin (fig. 23). As for digipurpurin, no clear-cut indication for the presence of the

corresponding bis- and monodigitoxosides could be obtained (cf. fig. 23);

d. There is a pronounced difference in the glycoside content of the gitoxin-peak, as calculated from the aglycone- and the desoxysugar-reactions, resp., which we encountered in leaf samples from the strains 15 and 20. Although our experiments strongly suggest that this phenomenon is to be ascribed to the presence of an isomer of gitoxin (e.g. with a $\Delta^{\beta:\gamma}$-lactone grouping), we did not succeed in isolating such an isomer;

e. Two digitanol glycosides, designated compounds OD1 and OD2, are present in the gitoxin-peak for samples from strain F (cf. fig. 24);

f. Digiproside appears close to strospeside in the distribution pattern. It principally occurs in leaf samples from young plants;

g. Several unknown glycosides, mainly belonging to the digitanol series, appear at the end of the distribution curves, with a maximum around tube 80. Since these glycosides are of minor importance quantitatively, we did not investigate these compounds any further.

CHAPTER IV.

In order to depend not only on paperchromatographic comparison, most of the compounds encountered in the various peaks of the distribution curves were isolated in pure form. To this end we made use of either adsorption chromatography on aluminium oxide or partition chromatography on columns of silicagel and cellulose powder, applied to appropriate fractions of the countercurrent distributions. The obtained substances were identified by comparison with authentic samples as to melting point, mixed melting point, R_F-values (both before and after hydrolysis to the genins and sugars), ultraviolet maxima and, occasionally infrared absorption spectra. When the amount of material was adequate, we also performed a measurement of the specific rotation and a C–H analysis.

The following substances were isolated and in most cases identified (the corresponding peak maxima are indicated in parentheses): compound OD3(7), digitoxin (22), digitoxigenin bis- and monodigitoxosides (28 and 34, resp.), gitaloxin (in a non-deformylated

leaf extract) (28), digipurpurin (39), gitoxin (54), gitoxigenin bis- and monodigitoxosides (60 and 66, resp.), compounds OD1 and OD2 (52 and 60, resp.) and strospeside (68).

Compound OD3 was probably formed from diginin during its isolation. As this is not rigorously proven, the designation "compound OD3" has been retained throughout this publication.

The melting points of our preparations of the digitoxigenin mono- and bisdigitoxosides were appreciably higher than published values, whereas our monodigitoxoside was some 5 to 15 degrees more dextrorotatory. We suggest the presence of small amounts of a related glycoside, probably the corresponding α-D-digitoxopyranoside, in the preparations of the monodigitoxoside from the various laboratories. Apart from this, molecular rotation differences indicate a β-glycosidal linkage for the digitoxose-residues in digitoxin and the mono- and bisdigitoxosides (table 11).

Compounds OD1 and OD2 probably are a tridigitoxoside and a bisdigitoxoside, resp., derived from a digipurpurogenin-like aglycone. For their infrared absorptionspectra, see fig. 26. Their aglycones could not be differentiated paper-chromatographically from the aglycone fraction of digipurpurin when hydrolysed under identical conditions.

The gitoxin preparations, isolated from various leaf samples did not show a diminished response to the dinitrobenzoate colour reaction (cf. table 13). The mother liquors from the crystallization also behaved normally in this respect.

The mono- and bisdigitoxosides of gitoxigenin were obtained in very small amounts only. Although they were not quite pure, their properties pointed to the correct composition.

A survey of R_F-values of both sugars and glycosides/aglycones in various solvent systems is given in table 13 and 14, respectively.

CHAPTER V.

The results of the investigation of the three strains are compared.

Distribution patterns for the crude extracts from the strains 15, 20 and F in their young, mature and flowering stages appear in figures 27 to 35. Glycoside contents, as calculated from the peak heights observed, are given in tables 17, 19 and 21.

The results of the spectrophotometric determinations of glycoside contents in fermented leaves of the strains 15, 20 and F in their various growth-stages are dealt with in tables 18, 20 and 22, resp.

For a comparison between aqueous (1 : 100) and 70 % ethanolic (1 : 10) leaf powder extracts regarding their glycoside contents, see table 16. The figures in these tables refer in part to determinations of the total content of genins of the A- and B-series (performed for the whole extract, as well as for the chloroform extract and the water-layer) and in part to determinations of the glycoside content with 3,5-dinitrobenzoate, picrate and xanthydrol reagents (for the chloroform extract only).

The water soluble fraction for all strains chiefly consists of gitoxigenin-glycosides; for strain F the proportion of A-glycosides in the mixture is somewhat higher than for the other strains. Besides the polar glycosides known to occur in the leaves (e.g. digitalinum verum, glucoverodoxin and, possibly, odorobioside G), in all stages of development several other gitoxigenin derivatives, more polar than digitalinum verum, are encountered. For all strains the content of these water soluble glycosides is about the same in the various growth stages.

For all strains the chloroform soluble fractions, although strongly differing from each other, did not show significant variations in composition during the period of growth covered by this investigation. As a consequence we may conclude that the characteristics of each strain are maintained during development. In the strains 15 and 20 the B-glycosides are preponderant; strain 15 in all growth stages contains more strospeside and less gitoxin than the otherwise closely allied strain 20. Strain F differs strongly from the two other strains; in all growth stages digitoxin is the dominating glycoside in the chloroform extract. This strain is often used as starting material in the industrial preparation of digitoxin; to this end it is cultivated on a large scale in the Netherlands. Another strain of this type, not included in this investigation, is the so-called strain Cambridge[154]. The three strains studied show only minor differences in their content of digitanol glycosides.

The possibility of obtaining, by selective breeding, strains of Digitalis purpurea with a widely differing composition of their glycoside mixtures (particularly apparent in the chloroform soluble fractions), is confirmed by our investigation and the chemical characteristics of each strain are sharply defined.

Although the number of growth stages we investigated was rather small, several pertinent facts regarding changes in the composition of the glycoside mixture during growth are notable:

a. The total content of glycosides as well as the content of chloroform soluble glycosides is highest in the mature plants, lowest in the young plants and has an intermediate value in the flowering stage. Previous observations of other authors thus are confirmed. At the same time it follows that these differences in content are not caused by variations in the composition of the glycoside mixture during development of the plants;

b. With respect to the total glycoside content the water soluble fraction is steadily decreasing during development of the plants. As the principal sugar in the chloroform soluble fraction is digitoxose (+glucose), in the water soluble fraction on the contrary digitalose (+glucose), this shift in the composition should perhaps be interpreted as an enhancement of the synthesis of digitoxose relative to that of digitalose in the later stages of development (cf. LEMLI[142]). The occurrence of digiproside notably in young leaves could also be explained along these lines;

c. Changes in the composition of the glycoside mixture during growth do not follow strictly parallel paths, although a good deal of agreement exists;

d. In the young and to a lesser extent in the flowering plants, the digitanol glycosides constitute a relatively greater part of the total glycosides than in the mature plants. This finding agrees very well with the recently published hypothesis (TSCHESCHE[139]) that the digitanol glycosides might be precursors in the biosynthesis of the cardiac glycosides. Unfortunately, it cannot be taken as rigorous proof, since other explanations fit the observed differences equally well.